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Production of Dehydroamino Acid-Containing Peptides by *Lactococcus lactis*▽

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Nisin is a pentacyclic peptide antibiotic produced by some *Lactococcus lactis* strains. Nisin contains dehydroresidues and thioether rings that are posttranslationally introduced by a membrane-associated enzyme complex, composed of a serine and threonine dehydratase NisB and the cyclase NisC. In addition, the transporter NisT is necessary for export of the modified peptide. We studied the potential of *L. lactis* expressing NisB and NisT to produce peptides whose serines and threonines are dehydrated. *L. lactis* containing the *nisBT* genes and a plasmid coding for a specific leader peptide fusion construct efficiently produced peptides with a series of non-naturally occurring multiple flanking dehydrobutyrines. We demonstrated NisB-mediated dehydration of serines and threonines in a C-terminal nisin(1-14) extension of nisin, which implies that also residues more distant from the leader peptide than those occurring in prenisin or any other lantibiotic can be modified. Furthermore, the feasibility and efficiency of generating a library of peptides containing dehydroresidues were demonstrated. In view of the particular shape and reactivity of dehydroamino acids, such a library provides a novel source for screening for peptides with desired biological and physicochemical properties.

Lantibiotics are (methyl)lanthionine-containing antibiotics (4). Lantibiotics contain (methyl)lanthionines, i.e., thioether-containing amino acids, which are posttranslationally introduced by enzymes coded for by genes of the lantibiotic's operon. These enzymes dehydrate serine and threonine residues, thereby converting them into dehydroalanine and dehydrobutyrine residues, respectively. Subsequently, (methyl)lanthionines are formed by enzymatic coupling of the dehydroresidues to cysteines. Functional reconstitution of in vitro activity of one bifunctional enzyme, lacticin M (27), and of the cyclase NisC (14) has been achieved. The structure of NisC has also been determined (14), providing detailed information about its catalytic mechanism.

The pentacyclic lantibiotic nisin is produced by some *Lactococcus lactis* strains. Nisin is ubiquitously applied as a food preservative that inhibits the growth of mainly gram-positive spoilage and pathogenic bacteria. By binding to the pyrophosphate of lipid II with its N-terminal region, which encompasses the A and B rings, nisin exerts two different antimicrobial activities (1, 7). First, a transmembrane pore composed of both nisin and lipid II causes dissipation of the transmembrane ion gradients. Second, binding of nisin to lipid II causes cell killing by inhibition of cell wall synthesis. Nisin is composed of four methyllanthionines, one lanthionine, two dehydroalanines, one dehydrobutyrine, and twenty-six unmodified amino acids (6, 12). The dehydration reactions are carried out by the nisin dehydratase NisB. Replacement of the dehydroalanine at positions 5 of nisin (2) and subtilin (15) very much reduces the capacity to inhibit the outgrowth of spores. NisB can also dehydrate serine and threonine residues in peptides that are not at all related to lantibiotics such as, for instance, human peptide hormones, provided that these peptides are N terminally fused to the lantibiotic leader peptide (10). The presence of dehydroresidues in a nonlantibiotic peptide can influence the peptide's activity (16, 18, 21, 22, 25, 26), and they could be excellent starting points for chemical modification because of their reactivity. We have studied here the capacity of *L. lactis* cells containing the NisBT proteins to dehydrate and export model peptides, among which are semirandomized hexapeptides (Fig. 1). Our data indicate a high degree of versatility of NisB to dehydrate serine and threonine residues in peptides.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids in this study are listed in Table 1.

Molecular cloning. Nisin leader constructs (Fig. 1) were made by PCR-amplifying leader peptide-encoding plasmid, pNZnisA-E3, using a phosphorylated primer with a (nonannealing) peptide-encoding tail. DNA amplification was carried out using Phusion DNA polymerase (Finnzymes, Finland). Ligation was carried out with T4 DNA ligase (Roche). Electrotransformation of *L. lactis* was done as described previously (8) using a Bio-Rad gene pulser (Bio-Rad, Richmond, CA). Nucleotide sequence analysis was performed by BaseClear (Leiden, The Netherlands).

Culturing. *L. lactis* was grown at 30°C in M17 broth (24) supplemented with 0.5% glucose (GM17) or minimal medium (19) with or without chloramphenicol (5 μg/ml) and/or erythromycin (5 μg/ml). The concentration of the antibiotics was reduced to 4 μg/ml when both were present simultaneously. Prior to mass spectrometry, cells were cultured, and samples were prepared as follows. Overnight cultures of *L. lactis* NZ9000 grown in GM17 broth containing 4 μg of antibiotics/ml were diluted 1/100. After growth to an optical density at 600 nm of 0.4, cells were centrifuged, and the medium was replaced by minimal medium supplemented with 1/1,000 volume of filtered (0.45-μm pore size) overnight in *L.*

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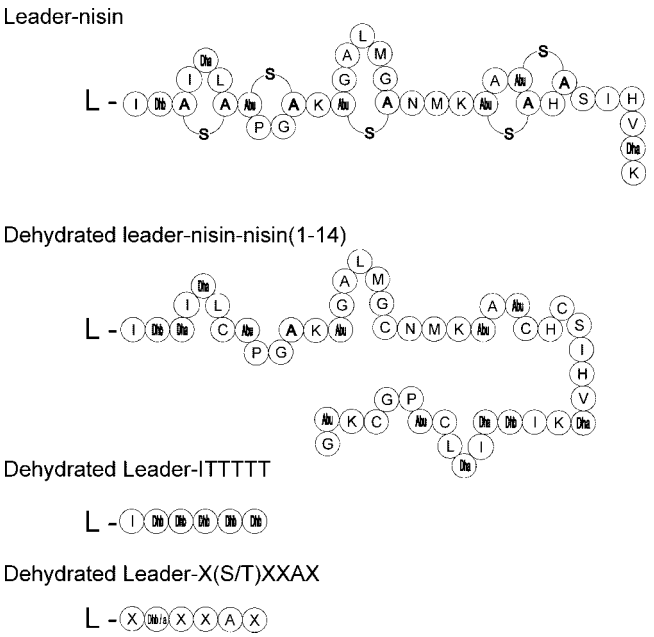

FIG. 1. Leader peptide fusions and their potential modifications. The nisin leader peptide (L) with the sequence MSTKDFNLDLVSV SKKDSGASPR directs the export and modification of peptides that are fused at its C terminus. This lantibiotic contains dehydroalanine (Dha), dehydrobutyrine (Dhb), and the thioether residues lanthionine (Ala-S-Ala) and methyllanthionine (Abu-S-Ala). Lanthionine or methyllanthionine results from the coupling of dehydroalanine or dehydrobutyrine to cysteine, respectively. Abu is aminobutyric acid. X is at a randomized position and can represent any amino acid. Dehydrated nisin-nisin(1-14) lacks one dehydration at an undefined site.

lactis NZ9700 culture medium containing nisin for induction. Incubation was continued overnight.

Mass spectrometry. Peptides were isolated from culture supernatants in a single step by applying the Ziptip procedure (C18 Ziptip; Millipore). Ziptips were wetted and equilibrated with 50% acetonitrile, followed by demineralized water. Subsequently, peptides from the medium were bound by subjecting 100 μl or more to Ziptip treatment, washed with 0.1% trifluoroacetic acid (TFA), eluted with a solution of 0.1% TFA with 50% acetonitrile, vacuum dried, and stored at −20°C until analysis. The dried Ziptip eluent was resuspended in 5 μl of 50% acetonitrile containing 0.1% (vol/vol) TFA, and 1 μl was applied to the target. Subsequently, 1 μl of matrix (5 mg of α-cyano-4-hydroxycinnamic acid/ml in 50% acetonitrile containing 0.1% [vol/vol] TFA) was added to the target and allowed to dry.

Mass spectra were recorded with a Voyager-DE PRO matrix-assisted laser desorption ionization–time of flight mass spectrometer (Applied Biosystems). In order to maintain high sensitivity, an external calibration was applied.

Hydrophobicity analysis of flanking residues of serines and threonines. The hydrophobicity of individual amino acids was taken as the consensus values described by Eisenberg (5). The hydrohobicity of dehydroresidues was approached by taking the value of alanine. The hydrophobicity of lanthionine was approached by taking the average between cysteine and alanine. Analyses were based on the peptides listed in Table 3, on NisB-dehydrated peptides described previously (10, 19), and on the wild-type nisin molecule. The flanking residues of serines and threonines in the sequence ITSIS, which occurs in nisin and in constructs previously described (10), were counted only once, as were also the amino acids D and R as flanking residues of serines and threonines in designed peptides. Residues that flank N- or C-terminal serines or threonines were not taken into account.

RESULTS AND DISCUSSION

NisB dehydrates multiple flanking threonines. We previously showed that NisB is equipped with a broad substrate specificity (10, 11, 19). To further evaluate the (lack of) fidelity of this enzyme, we investigated whether NisB can dehydrate multiple flanking threonines, which are not present in naturally occurring lantibiotics. Therefore, we expressed a construct, coding for the nisin leader extended with the amino acid sequence ITTTTT (Fig. 1 and Fig. 2), together with pIL3BTC encoding the nisin modifying and transporting enzymes. Mass spectrometric analysis of the culture supernatant demonstrated successful production of peptides. Partial N-terminal truncation of the leader part was observed as described previously (19). Up to fivefold dehydration was observed for the peptide devoid of the first five leader peptide residues, MSTKD (Fig. 2A). The mass peak of fourfold dehydration was most prominent, whereas the one of fivefold dehydration was small. Hydrophilic residues appear to disfavor dehydration (19). C-terminal serine residues are commonly not dehydrated in lantibiotics (19). Likely due to the hydrophilicity of the carboxy terminus, dehydration of the C-terminal threonine is inefficient. The fourfold dehydration could be clearly confirmed by four additions of ethanethiol (Fig. 2B). These data clearly demonstrate that NisB can dehydrate multiple flanking threonines, thus generating polydehydrobutyrine.

NisB is capable of dehydrating serine and threonine residues along the polypeptide chain. Ser33 of nisin is the dehydratable residue in the NisA peptide, which is most remote from the leader peptide, and has been reported to escape dehydration in ca. 10% of the cases (2, 20). The extent of modification of Ser33 was reduced to 50% in [Trp30]nisin A and [Lys27, Lys31]nisin A (9). Plasmid-encoded overproduction of NisB results in a higher extent of dehydration (9). To determine whether serine or threonine residues more distant from the leader peptide than position 33 can be dehydrated, the leader peptide-nisin construct was genetically C-terminally

TABLE 1. *Lactococcus lactis* strains and plasmids

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
NZ9000	*nisRK*$^+$	13
NZ9700	*nisABTCIPRKEFG*	13
pIL253-derived plasmids		23
pIL3BTC	*nisBTC*, derived from pIL2angBTC by deletion of the Em^r gene and introducing a stop codon at the start *ang* gene	19
pIL3BT	Derived from pIL3BTC; Cm^r	This study
pNZ8048-derived plasmids		13
pNZ-5T	Encodes leader-ITTTTT; Em^r	This study
pNZ-nisin-nisin(1-14)	Encodes leader-nisA-nisA(1-14); Em^r	This study
pNZnisA-E3	Nisin A; Em^r	11
pTPhexa	Encoding the nisin A leader, followed by a sequence encoding X(S/T)XXAX, in which X stands for a random amino acid; Em^r	This study

[a] Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance.

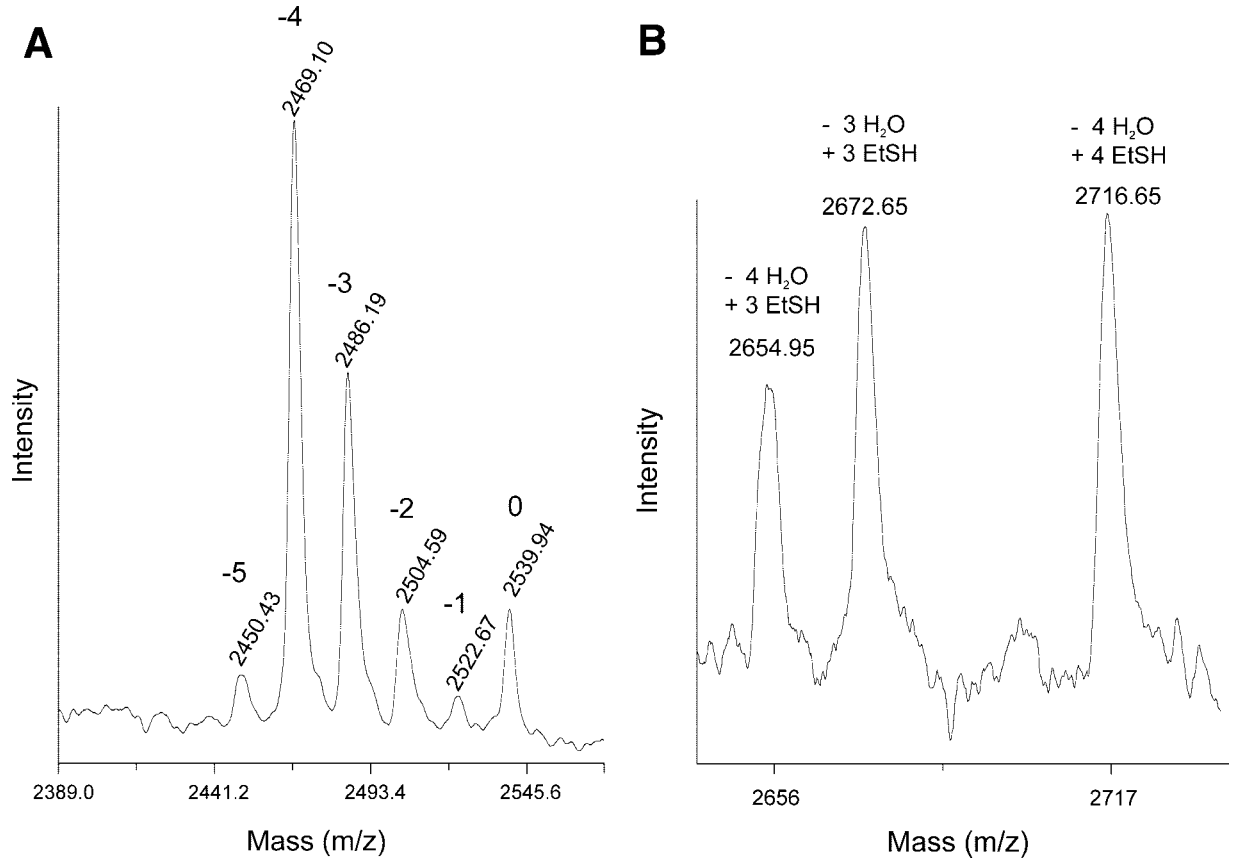

FIG. 2. NisB-mediated production of a peptide with five flanking dehydrobutyrines. A leader construct encoding the nisin leader peptide, followed by a sequence encoding ITTTTT, was expressed in *L. lactis* containing pIL3BTC. The supernatant was subjected to the Ziptip procedure, directly followed by mass spectrometry (A) or after ethanethiol (EtSH) treatment (B). Theoretical average mass ($M + H^+$) values for the peptide without the initial leader residues MSTKD, with numbers of dehydrations shown in parentheses, are 2,539.84 (zero), 2,521.82 (one), 2,503.81 (two), 2,485.79 (three), 2,467.78 (four), and 2,449.76 (five). The theoretical average mass values of the peptides without MSTKD in panel B are 2,672.19 (threefold dehydrated, three additions of ethanethiol), 2,654.18 (fourfold dehydrated, three additions of ethanethiol), and 2,716.32 (fourfold dehydrated, four additions of ethanethiol).

extended with nisin(1-14), the lipid II-binding domain of nisin. This yielded plasmid pNZ-nisin-nisin(1-14). In the supernatant of *L. lactis* cells containing pILnisBT and pNZ-nisin-nisin(1-14), a product with a mass peak of 6,979 Da ($M + H^+$) (Fig. 3) was clearly observed under reducing conditions in the presence of triscarboxyethyl phosphine (TCEP). This mass peak corresponded to a 12-fold-dehydrated fusion product of prenisin and nisin(1-14). These data indicate that in addition to the known dehydration steps in nisin, at least four of the five additional residues in the nisin(1-14) extension—Thr36, Ser37, Ser39, Thr42, and Thr47—can be dehydrated. Hence, NisB can also efficiently dehydrate residues that are more distant from the leader peptide than Ser33 in nisin. These modifications occur farther from the leader peptide than reported for any other known lantibiotic or model peptide, showing a high versatility of NisB.

A library of NisB-dehydrated peptides. We subsequently investigated the possibility of generating a library of peptides with dehydroresidues. Semirandomized primers were used to produce constructs that encode the nisin leader peptide fused to hexapeptides with four randomized positions. These hexapeptides were composed of X(S/T)XXAX, in which X represents any amino acid (Fig. 1 and Table 2). At least 75% of the obtained transformants produced peptides that could be detected by direct analysis of the supernatant by the matrix-assisted laser desorption ionization–time of flight method. Sequence analysis of plasmids of transformants that did not produce peptides with an expected mass revealed that some of these contained a stop codon at the start of—or within—the hexapeptide. Indeed, corresponding masses of the leader peptide without or with a few additional amino acids could be detected. Hence, the remarkable export of just the leader peptide itself, without propeptide fusion, had also occurred. Interestingly, most produced hexapeptides contained dehydrated residues, whereas some were completely devoid of dehydration (Table 2). The latter peptides contained mostly serine residues instead of threonine residues, a finding consistent with our previous prediction that serine is a less favorable substrate for NisB than threonine (19).

In agreement with the previously obtained in silico and in vivo data, hydrophobic flanking amino acid residues favor the dehydration of serine and threonine residues (Table 2). Exceptions are the hexapeptides VSPPAR, YTPPAL, and FSFFAF, which are not dehydrated. These sequences suggest

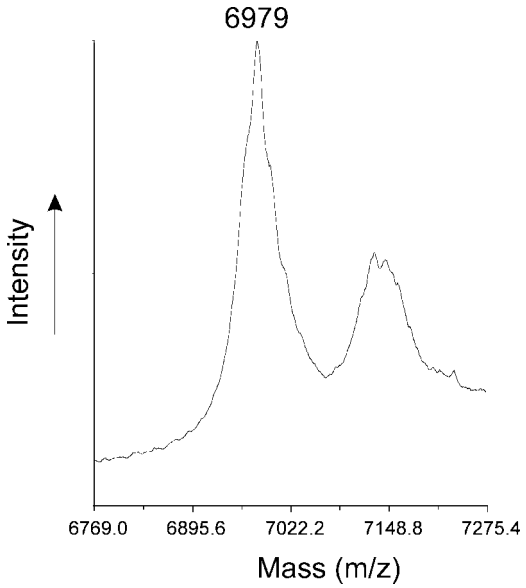

FIG. 3. NisBT-dependent production and excretion of dehydrated leader peptide-nisin-nisin(1-14). Nisin-induced *L. lactis* NZ9000 containing pNZnis-nis(1-14) and pIL3BT was cultured overnight in 100 ml of minimal medium, and the supernatant of the cells was then subjected, after centrifugation, to trichloroacetic acid precipitation; resuspended in 4 μl containing 5 mg of TCEP/ml; and analyzed by mass spectrometry. The expected average mass ($M + H^+$) for 12-fold-dehydrated leader peptide-nisin-nisin(1-14) without Met1 is 6,979 Da.

TABLE 3. Hydrophobic flanking residues favor NisB-mediated dehydration of serines and threonines[a]

Location	Mean hydrophobicity ± SD	No. of amino acids with:		Total no. of amino acids
		Positive hydrophobicity	Negative hydrophobicity	
N terminal of dehydrated S/T	0.40 ± 0.96^{A}	48	9	57
C terminal of dehydrated S/T	0.13 ± 1.12^{B}	39	18	57
N terminal of unmodified S/T	-0.36 ± 1.14^{C}	6	7	13
C terminal of unmodified S/T	-1.03 ± 1.33^{D}	4	9	13

[a] The mean hydrophobicity and corresponding standard deviations (σ_n) were calculated for amino acids in four positions: N and C terminally of NisB-dehydrated residues and N and C terminally of residues that had completely escaped NisB activity. Analyses were based on the normalized consensus hydrophobicities of amino acids (5). Using the *t* test, differences were found between the value marked by superscript A compared to values marked by superscript C ($P < 0.05$) or D ($P < 0.001$) and between the value marked by superscript B and the value marked by superscript D ($P < 0.01$).

that the presence of proline residues at positions 3 and 4 or flanked at both sides by a bulky phenylalanine may be unfavorable for NisB-mediated dehydration. Other exceptions to our previously proposed guidelines are the hexapeptides HTDLAD, KSHYAM, and RTSHAA. These are dehydrated, whereas the modified residues are flanked by two hydrophilic residues. Since neither HTRRAE nor HSKQAG is dehydrated, it is currently unclear how the presence of a histidine affects NisB-mediated dehydration. Nevertheless, it is generally observed among the peptides that escape dehydration that the serine/threonine residues are flanked by hydrophilic residues. Some peptides containing multiple serine and/or threonine residues were only incompletely dehydrated. Taken together, our analysis of random X(S/T)XXAX hexapeptides indicates that hydrophobic flanking of serine or threonine favors dehydration, whereas hydrophilic flanking—especially negatively charged amino acids—disfavors dehydration. Indeed, in the case of dehydration, the average hydrophobicity of flanking residues of serines and threonines is positive (0.40 and 0.13, N and C side, respectively), and predominantly amino acids with positive hydrophobicity are flanking the dehydroresidues (Table 3). In contrast, in the case of the absence of dehydration, the average hydrophobicity of amino acids that flank unmodified serines or threonines is negative (−0.36 and −1.03, N and C sides, respectively), and more flanking amino acids with negative hydrophobicity are present than with positive hydrophobicity (Table 3). Importantly, most of the cells containing NisBT and the randomizing plasmid pTPhexa produced dehydroamino-acid-containing peptide showing the high degree of promiscuity of NisB and NisT.

In the present study we demonstrate that nisin dehydratase and nisin transporter synthesizing *L. lactis* can produce a variety of dehydroresidue-containing peptides. Dehydration ap-

TABLE 2. NisB-mediated production of dehydrated semirandomized (hexa)peptides fused to the nisin leader[a]

No. of hydrophobic flankings	Codon sequence(s) at a dehydration of:			
	0	0/1	1	2
Two or three	VSPPAR, YTPPAL, FSFFAF	LSLPAA, LSANAG, VSNRA**S**, GTVRA**S**	VTLR*, ISARAD, ET**P**TAK, HS**P**TAG, NTLRA**S**, VSLLAR, L**T**AEAR	FTV**S**AR, AT**T**LAL, PS**T**IAI, AT**K**GLP**S**RHVLL*, R**STSAS**, AS**T**PAW
One	PSREAW, DSRKA**T**	RTVAAV, RTVAAG, WSELAG, **TS**NHA**S**, ISREAF	PTNVAG, PTRPAW, PTSRAF, PTRDAL, RTWPAK, NS**T**LAI	**TTS**AAK, VT**T**HAI, CTSVAF, **SST**AAF
None	RTQQAL, **SS**KLAR, HTRRAE, HSKQAG, RSKLAE	HTDLAD, KSHYAM		RTSHAA

[a] Using semirandomized primers, codons for the sequence X(S/T)XXAX were cloned behind the nisin leader, in which X represents any amino acid. NZ9000 containing pTPhexa and pIL3BTC was cultured, and its medium was analyzed by mass spectrometry as described in Materials and Methods. *, obtained as a result of a stop codon or a mutation. The propeptide parts of the obtained leader peptide fusions are depicted. S's and T's are indicated in boldface.

pears to be neither restricted by the presence of a series of flanking threonine residues nor restricted by the distance between the modifiable position and the nisin leader peptide. Previously, we showed that the threonine in a peptide composed of the nisin leader followed by the sequence DSRWA RVALIDSQKAAVDKAITDIAEKL was the only modified residue (19). Apparently, if NisB processively scans the substrate, then it does not release it even when none of the first 21 propeptide residues can be dehydrated. Processive dehydration has been observed in the case of the bifunctional lantibiotic enzyme LctM (17). These results raise intriguing questions regarding how NisB interacts with the nisin leader and the substrate domain. NisB is active in the absence of NisT and NisC (11), and therefore recognition of the substrate should be the consequence of a direct interaction of the leader peptide with NisB. Possibly, the substrate region slides along the active center of NisB, but it is unclear whether the leader peptide remains associated with NisB during catalysis and how it is transferred to NisC. Our previous studies have shown that NisC is not needed for peptide release from NisB, since dehydrated peptides can be excreted in the medium even in the absence of NisC. In view of the relative high hydrophobicity of the flanking residue at the N side of the dehydratable residue, it is tempting to speculate on a directionality of NisB-mediated dehydration of the substrate region from proximal to distal to the leader peptide. Mechanistic information on lantibiotic-enzyme-mediated dehydration is only available for LctM, a bifunctional enzyme that performs both the dehydration and the cyclization reaction. LctM phosphorylates serine and threonine residues prior to dehydration (3). It should, however, be noted that LctM has no sequence homology with NisB. The data presented here involving the dehydration of multiple flanking threonines, the dehydration of distal serines and threonines, and the library of dehydroresidue-containing peptides consistently indicate the great versatility of NisB. (Poly-) dehydrobutyrine sequences can be very interesting starting points for the synthesis of other non-natural amino acid residues or serve as coupling sites in further organic synthesis. Moreover, since dehydroresidues can be important constituents of biologically active peptides due to their particular planar shape and reactivity (16, 18, 21, 22, 25, 26), screening of a dehydroresidue-containing peptide library for desired properties and activities is a realistic and attractive possibility.

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